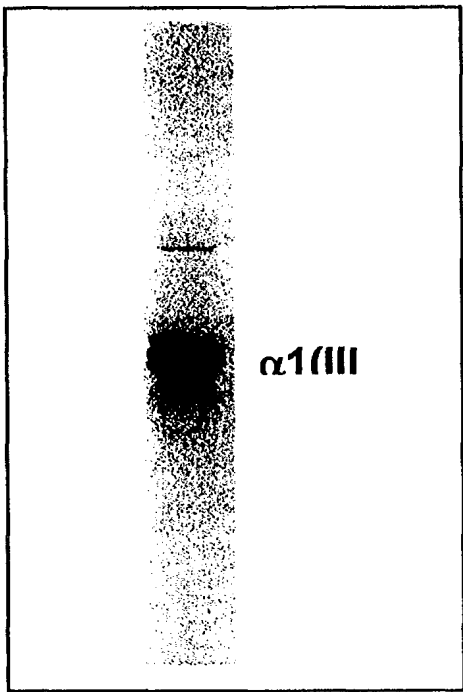




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<b>(21) International Application Number:</b> PCT/US99/18095 <b>(22) International Filing Date:</b> 10 August 1999 (10.08.99)  <b>(30) Priority Data:</b> 60/095,997                      10 August 1998 (10.08.98)                      US  <b>(71) Applicant:</b> FIBROGEN, INC. [US/US]; 225 Gateway Boulevard, South San Francisco, CA 94080 (US).  <b>(72) Inventors:</b> YANG, Chunlin; 1467 Marlin Avenue, Foster City, CA 94404 (US). POLAREK, James, W.; #3, A Dock, Waldo Point Harbor, Sausalito, CA 94965 (US). NEFF, Thomas, B.; 190 Glenwood Avenue, Atherton, CA 94027 (US).  <b>(74) Agents:</b> LAPIZ, Mariette, A. et al.; FibroGen, Inc., Legal Department, 225 Gateway Boulevard, South San Francisco, CA 94080 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> COLLAGEN TYPE I AND TYPE III HEMOSTATIC COMPOSITIONS FOR USE AS A VASCULAR SEALANT AND WOUND DRESSING		
<b>(57) Abstract</b> <p>Polymerized type I and/or III collagen based compositions for medical use as vascular sealants and wound dressings, and the preparation thereof, are described. Prior to polymerization, the collagen monomers are prepared recombinantly whereby chemical modifications of the collagen are not needed to form such monomers. The type I and/or III collagen compositions, which include gelatin comprised of type I and type III collagens, are useful as medical adhesives for bonding soft tissues and as sealants for a variety of medical uses. In a further aspect of the present invention, the polymerized type I and/or III collagen compositions include agents which induce wound healing or provide for additional beneficial characteristics desired in a tissue sealant or wound dressing. The compositions of the present invention are also useful in non-adhesive form.</p> <div style="text-align: right; margin-top: 100px;">  </div> <p style="text-align: center; margin-top: 20px;"><b>SDS-PAGE of RhC III from <i>Pichia</i></b></p>		

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## **COLLAGEN TYPE I AND TYPE III HEMOSTATIC COMPOSITIONS FOR USE AS A VASCULAR SEALANT AND WOUND DRESSING**

10

### **CROSS REFERENCE TO RELATED APPLICATIONS**

This application is a continuation-in-part of U.S. application provisional no.

15 60/095,977

### **FIELD OF THE INVENTION**

The present invention is directed to polymerized recombinant type I and/or type  
III collagen-based compositions and combinations thereof, including gelatin-based  
20 compositions, for medical use as sealants and wound dressings. The present invention is  
further directed to the preparation of such compositions. These compositions are useful  
as sealants in a variety of medical applications, including vascular plug type devices,  
wound closure devices, tendon wraps for preventing the formation of adhesion following  
surgical procedures, and dressings for use to treat incisions, seeping wounds, and the like,  
25 and as medical adhesives for bonding tissues. In a further aspect of the present invention,  
the compositions include agents which induce wound healing or provide additional  
beneficial characteristics desired in a tissue sealant. More particularly, the compositions  
of the present invention are can be used as vascular sealants.

30 

### **BACKGROUND OF THE INVENTION**

*Mechanical, Chemical, Synthetic and Autologous Adhesion Techniques.* The  
ability to bond biological tissues is an important area of investigation for biomedical  
researchers. Attempts to provide desired adhesion through purely mechanical bonding  
have proven to be neither convenient nor permanent. (Buonocore, M., Adhesion in  
35 Biological Systems, R. S. Manly, ed., Academic Press, New York, 1970, Chap. 15.) For  
example, the conventional methods of choice to close incisions in soft tissue following

5 surgery, injury, and the like have been sutures and staples. These techniques and methods are limited by, for example, tissue incompatibility with sutures or staples which may cause painful and difficult to treat fistulas granulomas and neuromas. Mechanical means can also be limited to being purely adhesive, and thus not fully satisfactory as compared to sealants, applied to close wounds that are bleeding or seeping, etc. Sutures  
10 and staples may also tend to cut through weak parenchymatous or poorly vascularized tissue. Sutures can leave behind a tract which can allow for leakage of fluids and organisms. Sutures can be further problematic in that the needle for any suture is larger than the thread attached to it and the needle tract is thus larger than can be filled by the thread used to form the sutures.

15 In addition, limits are imposed by the manual and visual dexterity required on the part of the surgeon and the excessive amount of time needed for the use of sutures or staples in microsurgeries. Furthermore, the joints in the gaps between staples or sutures, even when properly applied, the staples or sutures are inherently weak or may structurally weaken over time and leak.

20 Several investigators have worked on laser closure of wounds. (See, e.g., Abergel, R.P. et al. (1986) *J. Am. Acad. Dermatol.* 14(5):810-814; Cespanyi, E. et al. (1987) *J. Surg. Res.* 42(2):147-152; Oz, M.C. et al. (1989) *Lasers Surg. Med.* 9(3):248-253; Oz, M.C. et al. (1991) *Am. Surg.* 57(5):275-279; and Oz, M.C. et al. (1993) *J. Clin. Laser Med. Surg.* 11(3):123-126.) Early efforts concentrated on welding tissues using  
25 lasers of different wavelengths applied directly to wound edges and investigating the microstructural basis of the tissue fusion thus produced. Researchers proposed that a homogenizing change in collagen with interdigitation of altered individual fibrils. (See, e.g., Schober, R. et al. (1986) *Science* 232(4756):1421-1422.) Investigators explored the idea of heating the collagen fibrils above a threshold level allowed for cross-linking.  
30 However, the heat necessary to allow this reaction causes collateral thermal damage. This is undesirable as even a slight distortion in, for example, ocular tissue may have functional consequences. Also, in the event of laser weld failure, the edges of the tissues may be damaged by the original treatment and cannot be re-exposed to laser energy.

5 Further research attempted to enhance heat-activated cross-linking by placing a dye in the wound. It was reported that matching the absorbance of the dye with the laser wavelength achieved an adhesive effect with less laser power output and collateral thermal injury. (See, e.g., Chuck, R.S. et al. (1989) *Lasers Surg. Med.* 9(5):471-477; and Oz, M.C. et al. (1990) *J. Vasc. Surg.* 11(5):718-725.) Coupling the dye with a protein to  
10 create a tissue "solder" was also investigated. The protein commonly used is fibrinogen, and, in particular, autologous fibrinogen, which is used to avoid problems of the transfer of viral diseases through the use of blood components from pool donors. In previous applications, fibrinogen has been obtained as a fraction of whole blood, contains other blood elements, such as clotting factors. Application of such a protein-dye mixture in  
15 various animal models proved to be an improvement to dye alone. (See, e.g., Moazami, N. et al. (1990) *Arch. Surg.* 125(11):1452-1454; and Oz et al. (1990).) However, direct application in humans was prevented due to the need to isolate the necessary protein fibrinogen from the patient prior to the procedure to avoid risks of infection from donor plasma. Other proteins, for example, albumin, were unsatisfactory substitutes as welds of  
20 comparable strength were not achieved.

Comparisons of protein-dye applications and sutured closures show that the protein-dye applications produce less of an inflammatory response and result in greater collagen production, greater mean peak stress at rupture, and better cosmesis. (See, e.g., Wider, T.M. et al. (1991) *Plast. Reconstr. Surg.* 88(6):1018-1025.) Ophthalmologic  
25 applications of such a tissue solder have included the sealing of conjunctival blebs, sclerostomy, closure of retinectomies, and thermokeratoplasty. (See, e.g., Fink, A.J. et al. (1986) *Am. J. Ophthalmol.* 101(6):695-699; and Latina, M.A. et al. (1990) *Arch. Ophthalmol.* 108(12):1745-1750.)

Due to the deficiencies and limitations of mechanical means, such as the above  
30 mentioned sutures, staples, and laser techniques, efforts were made to develop synthetic polymers, such as, for example, cyanoacrylates, as biomedical adhesives and sealants. These plastic materials, however, induce inflammatory tissue reactions. In addition, the ability of these materials to establish permanent bonding under physiological conditions has yet to be fully realized.

5           The known toxicity associated with synthetic adhesives has led to investigators to the development of biologically-derived adhesives as bonding materials. Fibrin-based  
glues, for example, have commanded considerable attention. (See, e.g., Epstein, G. H. et  
al. *Ann. Otol. Rhinol. Laryngol.* 95: 40-45 (1986); Kram, H. B et al. *Arch. Surg.* 119:  
1309-1311 (1984); Scheele, J. et al. *Surgery* 95: 6-12 (January 1984); and Siedentop, K.  
10 H. et al. *Laryngoscope* 93: 1310-1313 (1983) for general discussion of fibrin adhesives.)  
Commercial fibrin tissue adhesives are derived from human plasma and thus pose  
potential health risks such as adverse immunogenic reactions and transmission of  
infectious agents, such as, for example, Hepatitis B virus. Moreover, the bond strength  
imparted by such adhesives are relatively weak compared to collagen adhesives. (See, for  
15 example, De Toledo, A. R. et al. *Assoc. for Res. in Vision and Ophthalmology, Annual  
Meeting Abstract, Vol. 31, 317 (1990).*) Accordingly, there is a need for safe and  
effective biologically compatible tissue adhesives for biomedical applications.

More recently, combination products have been devised for use as tissue  
adhesives and sealants. The use of a combination of three separately prepared substances,  
20 human fibrinogen cryoprecipitate, thrombin in the presence of calcium ion, and Factor  
XIII concentrate, to obtain a glue for application in skin graft applications,  
myringoplasty, repair of dural defects, hemeostatis after tonsillectomy, and tracheoplasty  
has been described. (See, Staindl (*Ann. Otol* (1979) 88:413-418).) In this same time  
frame, Immuno-AG, Vienna, Austria, began producing and commercializing a two-  
25 component "fibrin seal" system, wherein one component contains highly concentrated  
human fibrinogen, Factor XIII, and other human plasma protein, prepared from pooled  
blood, and the other component supplies thrombin and calcium ion. The two components  
are added together in the presence of a fibrinolysis inhibitor. After application,  
coagulation and fibrin cross-linking occur. Eventually, the seal may lyse in the process of  
30 healing of the wound or trauma which accompanies the reconstruction of the tissue. The  
development of an applicator device for this system which mixes and applies the two  
components of the system simultaneously has been described. (Redl, H., et al.,  
"Biomaterials 1980," Winter, G. D., et al., eds. (1982), John Wiley & Sons, Ltd., at page  
669-675.) These combination systems and their uses have been described widely. (See,

- 5 e.g., Seelich, T., J Head and Neck Pathol (1982) 3:65-69; O'Connor, A. F., et al.,  
Otolaryngol Head Neck Surg (1982) 90:347-348; Marquet, J., J Head and Neck Pathol  
(1982) 3:71-72; Thorson, G. K., et al., J Surg Oncol (1983) 24:221-223.) It has also been  
reported that the addition of barium ion to this fibrin glue system in the treatment of a  
bleeding duodenal sinus facilitates follow-up surveillance. (See, for example, McCarthy,  
10 P. M., et al., Mayo Clin Pros (1987) 62:317-319; Portmann M., J Head and Neck Pathol  
(1982) 3:96; Panis, R., *ibid.*, 94-95.)

Efforts have recently focused on methods which seek to avoid health issues raised  
by the use of blood plasma-derived products in commercially available tissue adhesive  
products and systems. Attempts have been made to isolate an autologous counterpart of  
15 the fibrinogen-containing component. (See, for example, Feldman, M. C., et al., Arch  
Otolaryngol-Head and Neck Surg (1988) 114:182-185; Feldman, M. C., et al., Arch  
Ophthalmol (1987) 105:963-967; Feldman, M. C., et. al., M J Otolog (1988) 9:302-305;  
Silberstein, L. E., et al., Transfusion (1988) 28:319-321.) Use of autologous fibrinogen  
preparations also have obvious limitations.

20 ***Vascular Sealants.*** One critical aspect of tissue adhesion is the sealing of  
wounds, and, in particular, vascular punctures and other vascular wounds resulting from,  
for example, surgery.

For example, percutaneously accessing major vascular structures is a key step in a  
variety of diagnostic and therapeutic procedures, including Percutaneous Transluminal  
25 Coronary Angioplasty (PTCA), Percutaneous Coronary Angiography, and Percutaneous  
Coronary Atherectomy. In a percutaneous intravascular procedure, access to the vascular  
space is generally obtained using the so-called Seldinger technique where, first, a hollow  
needle is used to create a puncture wound through the skin, the underlying muscle tissue,  
and the wall of a selected blood vessel, such as the femoral artery. Next, a guidewire is  
30 inserted through the tubular needle until its distal end is located in the blood vessel, at  
which time the needle is stripped off of the guidewire and replaced with an introducer  
sheath and dilator. The introducer sheath typically includes a self-sealing hemostatic  
valve on its proximal end for sealing around the guidewire. The guidewire is then  
advanced into the vascular space through the introducer and directed to a preselected area

5 of the vascular system. Once the guidewire is positioned, a catheter is advanced over the guidewire to the desired area.

Once the procedure has been completed and the catheter and the introducer sheath are removed from the puncture site, there may be profuse bleeding, especially when the patient has been on anticoagulant therapy such as heparin, coumadin, aspirin, or  
10 thrombolytic agents. The most common method used to prevent post-procedure bleeding at the access site involves the application of direct pressure to the perforation site until normal physiologic pathways have sealed the access site. There are several problems with this method. First, the pressure application technique may fail to prevent hemorrhage. Such a hemorrhage may be life-threatening or can lead to a large  
15 hematoma. A large hematoma in the groin, for instance, may compromise the major nerve supply to the anterior lower extremity.

Secondly, the pressure application technique extends the length of the in-hospital stay. For example, a PTCA may be completed in 2 to 3 hours, but the patient will typically be hospitalized for several additional hours or overnight to allow the access site  
20 to seal physiologically. During this extended hospital stay the patient is required to stay immobile, often with a sand bag taped to the patient's thigh, such as in the case of femoral artery access.

These and other complications are exacerbated where PTCA procedures are performed in elderly patients who commonly have arteries with reduced natural elasticity.  
25 The access perforation in a relatively inelastic artery does not contract or shrink upon itself to the same extent as in an artery of normal elasticity. The resulting undeflected perforation is typically two to three times larger than an access perforation in a normal artery, further complicating the initiation of hemostasis and the normal physiologic sealing of the access site.

30 More than 500,000 PTCAs were performed worldwide in 1992 (Cowen Report, March 1993), and several times that number of other procedures requiring accessing major vascular structures percutaneously and were also performed. Thus, the increased length of in-hospital stay necessitated by the pressure application technique considerably increases the expense of procedures requiring such vascular access.



5           A technique that would allow faster and safer sealing of a vascular access site would save a significant amount of health care resources. Medical literature has addressed the problem of achieving hemostasis following removal of a percutaneously applied intravascular introducer in such uses as angiography or angioplasty by a number of divergent means. U.S. Patent No. 5,290,310 describes a device for delivering a  
10 collagen plug subcutaneously against a penetration site in a wall of a blood vessel. An instrument containing a toroidal-shaped collagen plug within a barrel thereof is made to surround the exterior of a tubular introducer. The instrument includes a pusher mechanism for ejecting the collagen plug into the puncture wound and against the exterior wall of the blood vessel at the site of the puncture. This device relies upon a  
15 collagen plug which is derived from animal sources and is therefore comprised primarily of heterotrimer collagen type I.

          U.S. Patent No. 5,129,882 also discloses a surgical implement for injecting a hemostatic agent in a puncture wound by routing the injection device through the lumen of the introducer sheath after it has been retracted sufficiently so that the distal end  
20 thereof is no longer in the blood vessel. Deploying a plunger, the hemostatic agent is forced out of the instrument and against the exterior wall of the artery proximate the puncture wound.

          U.S. Patent Nos. 4,744,364, 4,852,974, 4,890,612, 5,021,059, and 5,222,974 each describe a method and apparatus for effecting hemostasis by inserting an anchoring  
25 device through the puncture wound and into the blood vessel while using a filament attached to the anchoring device to inject an appropriate sealant into the wound. The anchoring device prevents entrance of the sealing material into the blood vessel and serves as an anchor and guide for addressing selected vessels.

          Still other devices for injecting a hemostatic agent into a puncture wound  
30 following a vascular procedure are described in U.S. Patent Nos. 5,281,197, 4,838,280, 5,192,300, and 4,738,658 and in published European Patent Application 0 476 178A1.

***Collagen/Gelatin As A Biomaterial.*** Collagen, the major connective tissue protein in animals, possesses numerous characteristics not seen in synthetic polymers. Characteristics of collagen include good compatibility with living tissue,

5 promotion of cell growth, and absorption and assimilation of implantations. (See, e.g., Shimizu, R. et al. *Biomat. Med. Dev. Art. Org.*, 5(1): 49-66 (1977).) These same characteristics are also true of gelatins, derivation products of collagens.

Various applications of collagen as a biomaterial are being tested, for example, the use of collagens in dialysis membranes of artificial kidney, artificial cornea, vitreous  
10 body, artificial skin and blood vessels, hemostatic agents, soft contact lenses, and in surgery. (Sterzel, K. H. et al. *Amer. Soc. Artif. Int. Organs* 17: 293 (1971), Rubin, A. L. et al. *Nature* 230: 120 (1971), and U.S. Pat. No. 4,581,030, Dunn, M. et al. *Amer. Soc. Artif. Int. Organs* 17: 421 (1971), Krajicek, M. et al. *J. Surg. Res.* 4, 290 (1964), U.S. Pat. No. 4,215,200, U.S. Pat. Nos. 4,264,155; 4,264,493; 4,349,470; 4,388,428; 4,452,925,  
15 and 4,650,616, and Chvapil, M. et al. *Int. Rev. Conn. Tiss. Res.* 6: 1-61 (1973).)

Natural collagen fibers, however, are basically insoluble in mature tissues because of covalent intermolecular cross-links that convert collagen into an infinite cross-linked network. Dispersal and solubilization of native collagen can be achieved by treatment with various proteolytic enzymes which disrupt the intermolecular bonds and remove  
20 immunogenic non-helical end regions without affecting the basic rigid triple-helical structure which imparts the desired characteristics of collagen. (See, e.g., U.S. Pat. Nos. 3,934,852; 3,121,049; 3,131,130; 3,314,861; 3,530,037; 3,949,073; 4,233,360, and 4,488,911 for general methods for preparing purified soluble collagen.)

Various methods and materials have been proposed for modifying collagen to  
25 render it more suitable as biomedical adhesives. (See, e.g., De Toledo, A. R. et al. *Assoc. for Res. in Vision and Ophthalmology*, Annual Meeting Abstract, Vol. 31, 317 (1990); Lloyd et al., "Covalent Bonding of Collagen and Acrylic Polymers," *American Chemical Society Symposium on Biomedical and Dental Applications of Polymers*, Polymer Science and Technology, Vol. 14, Plenum Press (Gebelein and Koblitz eds.), New York,  
30 1980, pp. 59-84; Shimizu et al., *Biomat. Med. Dev. Art. Org.*, 5(1): 49-66 (1977); and Shimizu et al., *Biomat. Med. Dev. Art. Org.*, 6(4): 375-391 (1978), for general discussion on collagen and synthetic polymers.) In many instances, the prior modified collagen-based adhesives suffer from various deficiencies, including (1) cross-linking/polymerization reactions that generate exothermic heat, (2) long reaction times,

5 and (3) reactions that are inoperative in the presence of oxygen and physiological pH ranges. (See, e.g., Lee M. L. et al. *Adhesion in Biological Systems*, R. S. Manly, ed., Academic Press, New York, 1970, Chap. 17.) Moreover, many of the prior modified collagen-based adhesives contain toxic materials, rendering them unsuitable for biomedical use. (See, for example, Buonocore, M. G. (1970) and U.S. Pat. No.  
10 3,453,222.)

Additionally, the use of collagen-based adhesives also presents immunological concerns as such adhesives have been derived from animal sources and typically bovine sources. Studies with respect to the use of such collagens as injectible devices have reported minor inflammatory responses. More recently, potential issues regarding the  
15 transmission of disorders to humans related to bovine spongiform encephalopathy ("mad cow disease") have focused attention, especially in Europe, to limiting the use of animal, and particularly, bovine-sourced materials.

Notwithstanding these deficiencies, certain collagen-based adhesives, reportedly having appropriate adhesive strength and utility in many medical applications,  
20 particularly involving soft tissues, have been described. (See, e.g., U.S. Patent Nos. 5,219,895, 5,614,587, 5,582,834, 5,575,997, 5,354,336, and 4,600,574.) The literature identify the use of type I and type II in collagen-based adhesives wherein purified collagen types I and II are chemically modified to form monomers soluble at physiological conditions and polymerized to form compositions having adhesive and  
25 sealant properties. Notably, the reports are limited to collagen-based adhesives composed of collagens derived from natural sources which represent a collagen mixture. For example, type I collagen as isolated from natural sources typically contains approximately 10-20% type III and other collagens, depending upon the tissue source used, and about 90-80% type I collagen. With respect to the "collagen type I" mixtures,  
30 the literature further teaches only the use of collagen as an adhesive as a consequence of its structural characteristics, or, alternatively, the use of predominantly collagen type I heterotrimers, as compared to collagen type I homotrimers which have been implicated in the epithelial cell attachment. (See, e.g., Gherzi, et al., 1989, *Eur. J. Cell Biol.* 50:279-84)).

5 Available reports do not refer to collagen type III, the unexpected hemostatic characteristics of type III collagen, or the use of recombinant collagens which would allow the first chemical modification step, as described in the art, to be avoided.

In summary, there is a need in the art for compositions useful as sealants and wound dressings that permit faster and safer healing, that minimize the risks of infection  
10 from donor, including non-human, sources, that increase convenience and permanence, that minimize demands on surgical resources and time, and that demonstrate superior biocompatibility. In addition, there is a need for biologically-derived adhesives that offer improved convenience and permanence over currently available formulations, and that promote less-invasive treatment, resulting in improved patient comfort and shorter time  
15 under medical supervision. Additionally, such compositions would preferably offer improved bond strength. There is also a need for non-adhesive compositions that provide the above-named advantages of safer and more effective healing and that are biologically compatible.

## 20 SUMMARY OF THE INVENTION

The present invention includes biologically compatible, collagen type III and/or type I products with sealant properties which can be formed using soluble recombinantly derived collagen type III and/or type I monomers or gelatin derived from collagen type III and/or type I monomers (the collagen and gelatin products are collectively hereinafter  
25 referred to as "collagen") wherein said monomers are polymerized to form a collagen type III and/or type I composition having sealant properties. Preferably, the collagen is human and is derived using recombinant technology. Collagen type III was selected for its unexpectedly superior hemostatic characteristics, as compared to other collagen types. Collagen type I was selected for its structural characteristics, as well as for the hemostatic  
30 properties of certain collagen type I forms (e.g., collagen type I homotrimers). The polymerization reaction may be initiated with an appropriate polymerization initiator such as a chemical oxidant, ultraviolet irradiation, a suitable oxidative enzyme, or atmospheric oxygen. Additionally, cross-linking agents including glutaraldehyde, dye-mediated photooxidation, PEG and its derivatives, acyl azide, polyepoxy fixatives,

5 oxidized starch (periodate) and water soluble carbodiimide ("WSC") may be used in the polymerization process to form a collagen composition having sealant properties.

For purposes of optimizing the sealant and adhesive properties of the recombinant collagen product by optimizing the structural stability and the hemostatic characteristics of the product, the product is comprised of a combination of pure recombinant type I and type III collagen. The ratio of pure recombinant collagen type III to pure recombinant collagen type I (heterotrimer) is preferably about 30% and greater type III collagen to about 70% or less type I collagen (heterotrimer). More preferably, the ratio of pure recombinant type III collagen to pure recombinant type I collagen (heterotrimer) is about 30% to about 50% type III collagen to about 70% to about 50% type I collagen (heterotrimer). Most preferably, the ratio of pure recombinant type III collagen to pure recombinant type I collagen (heterotrimer) is about 30% to about 40% type III collagen to about 70% to about 60% type I collagen (heterotrimer).

With respect to compositions comprised of collagen type I homotrimer, the ratio of pure recombinant type I homotrimer to a combination of recombinant collagen type I heterotrimer and recombinant collagen type III is about 90:10. More preferably, the ratio of pure recombinant type I homotrimer to a combination of recombinant collagen type I heterotrimer and recombinant collagen type III is about 75:25. Most preferably, the ratio of pure recombinant type I homotrimer to a combination of recombinant collagen type I heterotrimer and recombinant collagen type III is about 50:50.

25 It is the object of this invention to provide for a pure recombinant collagen type III tissue sealant, a pure recombinant type I tissue sealant, or a pure recombinant collagen type I and type III tissue sealant, free from other collagen types, having at least one of the following characteristics and capabilities:

(i) Hemostasis. The sealant acts as a hemostatic barrier and reduces the risk of serum, lymph, and liquid leakage. As collagen type III possesses inherently hemostatic properties, its use in a hemostatic device provides an improvement over known fibrin sealants. Collagen type I also possesses some hemostatic properties.

(ii) Gluing. Due to its adhesive properties, the sealants of the present invention connect tissues by forming a strong joint between them and adapt uneven

5 wound surfaces. The glueing effect is increased by a combination of agents, such as those described below, and collagen type III and/or collagen type I.

(iii) Wound healing. The sealant promotes the growth of fibroblasts which, in combination with efficient hemostasis and adhesion between the wound surfaces provides for an improved healing process. (See also, Ghersi, et al., 1989, Eur. J. Cell Biol. 50:279-284 (comparing characteristics of homotrimer and heterotrimer collagen type I).) The use of the present compositions as anti-adherence/wound healing compositions is expected to result in a normal (regenerative) tissue rather than scar tissue, i.e. optimal wound healing. Furthermore, such compositions also reduce the inflammatory response.

15 Accordingly, it is an object of the present invention to provide polymerized collagen type III and/or type I compositions as safe, effective biological adhesives with appropriate adhesive strength for biomedical applications, particularly those involving soft tissues. More specifically, the present invention is directed to compositions useful in sealing punctures and incisions in large blood vessels and the heart. The polymerized materials may assume a number of sizes and shapes consistent with their intended biomedical applications, which include use in ophthalmology, plastic surgery, orthopedics, and cardiology. The vascular sealant compositions of the present invention, comprising collagen type III and/or I, may be used alone or in combination with a tissue sealant device, including, for example, the devices set forth in U.S. Patent Nos. 5,782,860  
20 (issued July 21, 1998), 5,759,194 (issued June 2, 1998), and 5,728,132 (issued March 17, 1998).

In another object of the invention, the collagen type III and/or type I composition is further comprised of agents which will confer additional desirable characteristics for a vascular sealant or wound dressing. For example, fibrin, fibrinogen, thrombin, calcium ion, and Factor XIII may be included in the composition to better effect the formation of a three-dimensional network of polymerized collagen. In yet another object of the invention, the recombinant collagen type III composition incorporates a compound having wound healing capabilities. In one embodiment, the compound is connective tissue growth factor and is incorporated in the composition to effect slow-release of the  
30

- 5 compound to the wound. In a second embodiment of the invention, the drug improves vascularization, for example, tumour necrosis factor, as described in U.S. Patent No. 4,808,402 (issued February 28, 1989).

## BRIEF DESCRIPTION OF THE DRAWINGS

- 10 Figure 1 shows SDS-PAGE analysis of recombinant type III collagen produced by *pichia pastoris*.

Figure 2 shows data relating to the biocompatibility of recombinant type III collagen and a commercially available collagen hemostat.

- 15 Figure 3 shows data relating to platelet aggregation experiments of recombinant type III and bovine collagen type I.

Figure 4 shows data relating to the bleeding time of spleen treated with recombinant collagen type III and bovine collagen type I.

Figure 5 shows a SDS-PAGE analysis of bovine collagen I cross-linked with water soluble carbodiimide.

- 20 Figure 6 shows a SDS-Page analysis of recombinant collagen type III cross-linked with water soluble carbodiimide.

## DETAILED DESCRIPTION OF THE INVENTION

- It is understood that the present invention is not limited to the particular  
25 methodology, protocols, cell lines, vectors, and reagents, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly  
30 dictates otherwise. Thus, for example, a reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

- Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Preferred methods, devices, and materials are described, although any  
35 methods and materials similar or equivalent to those described herein can be used in the

5 practice or testing of the present invention. All references cited herein are incorporated by reference herein in their entirety.

### Definitions

As employed herein, the term "biologically compatible" refers to recombinant collagen type III and/or type I modified in accordance with the present invention (i.e., a  
10 polymerized collagen type III recombinant product) which is incorporated or implanted into or placed adjacent to the biological tissue of a subject and more particularly, does not deteriorate appreciably over time or induce an immune response or deleterious tissue reaction after such incorporation or implantation or placement.

As employed herein, the term "pure recombinant collagen type I" refers to  
15 collagen type I manufactured by recombinant techniques which is substantially free from other collagen types. Unless otherwise specifically referenced, the term pure recombinant collagen type I includes both collagen type I homotrimer and collagen type I heterotrimer and mixtures thereof. The term includes any other forms of recombinant collagen type I and any modifications made thereto that may be categorized as a subset of collagen, such  
20 as gelatins. The term excludes collagen type I isolated from natural sources.

As employed herein, the term "pure recombinant collagen type III" refers to human collagen type III manufactured by recombinant techniques which is substantially free from other collagen types. The term includes any other forms of recombinant collagen type III and any modifications made thereto that may be categorized as a subset  
25 of collagen, such as gelatins. The term excludes collagen type III isolated from natural sources.

As employed herein, the term "substantially free" refers to a recombinant collagen type that is substantially pure of any other collagen type or unmixed with any other collagen type, and is preferably at least 90% free from other collagen types.

30 As employed herein, the term "vascular sealant" refers to any composition useful in closing vascular wounds, including plugs, which possesses hemostatic properties.

As employed herein, the term "wound" refers to any opening in the skin, mucosa or epithelial linings, most such openings generally being associated with exposed, raw or abraded tissue. There are no limitations as to the type of wound or other traumata that



5 can be treated in accordance with this invention, such wounds including, but are not limited to, first, second, and third degree burns (especially second and third degree); surgical incisions, including those of cosmetic surgery; wounds, including lacerations, incisions, and penetrations; and ulcers, including decubital ulcers (bed-sores) and ulcers or wounds associated with diabetic, dental, haemophilic, malignant, and obese patients.

10 Although the primary concern is the healing of major wounds by neovascularization, it is contemplated that the present invention may also be useful for minor wounds, and for cosmetic regeneration of epithelial cells. Preferably, the wounds to be treated are burns and surgical incisions, whether or not associated with viral infections or tumors

### 15 **Preparation of Polymerized Recombinant Collagen Type I and III**

*Production of Collagen Type I and III Monomers.* Types of collagen useful in forming the biologically compatible collagen products of the invention with adhesive and hemostatic properties are recombinant collagen type I and type III. Monomeric soluble collagen types I and III is obtained by recombinant processes, including processes

20 involving the production of collagen type III in transgenic animals. Such recombinant processes are set forth, for example, in U.S. Patent No. 5,593,859, which is incorporated herein by reference. Preferably, collagen types I or III will be recombinantly manufactured by culturing a cell which has been transfected with at least one gene encoding the polypeptide comprising collagen type I or III and genes encoding the  $\alpha$  and

25  $\beta$  subunits of the post-translational enzyme prolyl 4-hydroxylase and purifying the resultant collagen monomer therefrom. Preferably, the monomeric soluble collagen type I and III material exhibits a viscous consistency and varying degrees of transparency and clarity.

*Polymerization Of Collagen Type I and III Monomers.* The recombinant

30 collagen type I and III solution may be subsequently subjected to polymerization or cross-linking conditions to produce the polymerized collagen composition of the present invention. Polymerization may be carried out using irradiation, e.g., UV, gamma, or fluorescent light. UV irradiation may be accomplished in the short wave length range using a standard 254 nm source or using UV laser sources. With a standard 254 nm

5 source, 4-12 watts, polymerization occurs from 10 to 40 minutes, preferably 20 to 30 minutes, at an exposure distance of from 2.5-10 cm, preferably from 2.5 to 5 cm distance. Excess UV exposure will begin to depolymerize the collagen polymers. Polymerization using gamma irradiation can be done using from 0.5 to 2.5 Mrads. Excess gamma exposure will also depolymerize collage polymers. Polymerization in the presence of  
10 oxygen can be achieved by adding an initiator to the fluid prior to exposure. Non-limiting examples of initiators include sodium persulfate, sodium thiosulfate, ferrous chloride tetrahydrate, sodium bisulfite, and oxidative enzymes such as peroxidase or catechol oxidase. When initiators are employed, polymerization occurs in 30 seconds to 5 minutes, usually from 1 to 3 minutes.

15 The polymerizing agent is preferably UV irradiation. However, the polymerization or cross-linking of the monomeric substituents can be carried out by any of the methods will known in the art, including simply exposing the material to atmospheric oxygen, although the rate of polymerization is appreciably slower than in the case of UV irradiation or chemical agents.

20 Other agents may also be useful in the polymerization process. For example, to improve the cohesive strength of adhesives formed from the compositions of this invention, difunctional monomeric cross-linking agents may be added to the monomer compositions of this invention to effect polymerization. Such cross-linking agents are known in the art, for example, in U.S. Pat. No. 3,940,362 which is hereby incorporated by  
25 reference herein.

Additionally, polymerization methods and cross-linking agents such as glutaraldehyde, dye-mediated photooxidation, PEG and its derivatives, acyl azide, plyepoxy fixatives; oxidized starch (periodate) and water soluble carbodiimide ("WSC") well known in the art may be used to produce the polymerized collagen composition of  
30 the present invention. (See, e.g., U.S. Patent No. 4,615,794, U.S. Patent No. 5,444,154, U.S. Patent No. 4,500,453, U.S. Patent No. 5,702,818, U.S. Patent No. 5,415,938, U.S. Patent No. 5,308,641, U.S. Patent No. 5,264,551, U.S. Patent No. 5,258,501, U.S. Patent No. 5,258,481, U.S. Patent No. 4,427,808, U.S. Patent No. 4,272,610.)

5           Moreover, the use of polyaldehyde compositions to effectuate polymerization can be also utilized. (See, for example, PCT WO 97/29715 and EP 747,066 A2.)

**Formation of Gelatin.** The recombinant collagen protein of the present invention may be further modified and processed into gelatin using procedures known in the art. (See, e.g., Veis, 1965, International Review of Connective Tissue Research, "The  
10   Physical Chemistry of Gelatin", Academic Press, New York and London.) For example, a common feature of all standard collagen to gelatin conversion processes is the loss of the secondary structure of the collagen protein, and in the majority of instances, an alteration in either the primary or tertiary structure of the collagen. The collagens of the present invention can be processed using different procedures depending on the type of  
15   gelatin desired.

          In one approach, modifications may occur to unpurified collagen or procollagen present in the cell mass or in the culture medium or any further modifications can be made to the purified collagen as described above. For example, recombinant collagen or procollagen may be modified and processed into recombinant gelatin. Gelatin may be  
20   produced directly from the cell mass or the culture medium by taking advantage of gelatin's solubility at elevated temperatures and stability at conditions of low or high pH, low or high salt concentration, and high temperatures. For example, the cell mass or culture medium may further be treated to extract gelatin by denaturing the triple helical structure of collagen using detergents, heat or denaturing agents. (See, e.g., Vies inter alia.) Operations well  
25   established for the manufacture of tissue-derived gelatin can be applied to the production of recombinant gelatin. This includes, but is not limited to, treatments with strong alkali or strong acids, heat extraction in aqueous solution, ion exchange chromatography, cross-flow filtration, and heat drying.

### 30   **Collagen Type I and III Compositions**

          The compositions of the present invention are comprised of polymerized type I and III collagen wherein said composition is manufactured by a process comprising the steps of: (1) production of collagen type I and III monomers by the recombinant methods described above; and (2) polymerization of such monomers. In addition, where the final

5 composition is a gelatin-based sealant or wound dressing, the process includes a step wherein the collagen is converted into gelatin.

For purposes of optimizing the sealant and adhesive properties of the recombinant collagen product by optimizing the structural stability of the product as well as the hemostatic characteristics of the product, the product is comprised preferably of a  
10 combination of pure recombinant type I and type III collagen. The ratio of pure recombinant collagen type III to pure recombinant type I (heterotrimer) is about 30% and greater type III collagen to about 70% or less type I collagen (heterotrimer). More preferably, the ratio of pure recombinant type III collagen to pure recombinant type I collagen (heterotrimer) is about 30% to about 50% type III collagen to about 70% to about  
15 50% type I collagen (heterotrimer). Most preferably, the ratio of pure recombinant type III collagen to pure recombinant type I collagen (heterotrimer) is about 30% to about 40% type III collagen to about 70% to about 60% type I collagen (heterotrimer).

The appropriate ranges of concentrations of components in the tissue sealants and adhesives of the present invention can be determined by methods well-known in the art.  
20 (See, e.g., Haraski, H. et al. (1999) Volume XXII, Society for Biomaterials, pages 158 through 159; Fasman, G. D., ed. (1989) Practical Handbook of Biochemistry and Molecular Biology, Section 1, pages 126 through 130; U.S. Patent No. 5,834,232 (issued November 10, 1998); Sierra, D. H. et al. (1992) J. Appl. Biomater. 3(2):147-151; Martinowitz, U. and R. Saltz (1996) Curr. Opin. Hematol. 3(5):395-402; and Sirieux, D.  
25 (1998) Ann. Vasc. Surg. 12(4):311-316.) The actual proportions of the collagen components of the compositions of the present invention will depend on the addition of other agents to the compositions and on the desired use of the compositions. The determination of suitable proportions for particular compositions is within the level of skill in the art, and this invention contemplates the various combinations that can be  
30 reached.

The compositions of the present invention may be further comprised of other agents useful in gluing or sealing vascular tissues, and more generally, soft tissue. For example, in addition to recombinant collagen type I and/or type III protein, the composition will preferably comprise transglutaminases such as Factor XIII and/or fibrin/

5     fibrinogen/fibronectin and/or plasminogen. The suitable concentrations of these components can be selected by methods well known in the art. For example, fibrinogen can be present in plasma concentrations, such as from about 1.5 to about 4.0 mg/ml, or higher. Fibrinogen can also be present in lower concentrations, for example, to monitor performance.

10           Preferably, the composition will also include clotting enzymes, i.e. thrombin, especially in combination with bivalent calcium, such as calcium chloride. The concentration of calcium chloride can vary, for example, from between 40 mM to 0.2 M, depending on the specific purpose of the tissue adhesive composition. High concentrations of calcium chloride inhibit fibroblast growth and are therefore preferred  
15     for anti-adherence applications (fibronectin, which stimulates the growth of fibroblasts, can be absent in such compositions). It may further be valuable to include a fibrinolysis inhibitor, such as a plasmin inhibitor, e.g. aprotinin, aprilotinin, alpha-2-antiplasmin, alpha-2-macroglobulin, alpha-1-antitrypsin, epsilon-aminocaproic or tranexamic acid, or a plasmin activator inhibitor, e.g., PAI-1 or PAI-2.

20           While the proportions of the previously known ingredients in the tissue adhesive compositions of the invention may be selected according to methods well-known in the art, the necessary amount of the viscosity-enhancing polymer can readily be determined by a person skilled in the art depending on the particular polymer and the intended use form. Thus, if the concentration and/or molecular weight of the viscosity-enhancing  
25     polymer is too low, the viscosity increase will be insufficient, and a too high concentration and/or molecular weight will inhibit the fibrin polymerization and the adhesion to the tissue.

          By increasing the thrombin concentration, the polymerization of composition of the present invention may be quickened, reducing the time until the glue sets. At low  
30     thrombin concentrations, for example, the fibrin of the composition will remain more or less fluid for several minutes after application. A further beneficial effect of increasing the viscosity with a viscosity-enhancing polymer in accordance with the invention is therefore that lower concentrations of thrombin, required in situations where the parts to be sealed require subsequent adaptation even on non-horizontal surfaces, can be used.

5           Likewise, the compositions of the present invention may, rather than including a combination of the agents described herein, include a fusion protein wherein the collagen type I and/or type III and, for example, fibrin, are combined to form one molecule. Such fusion proteins may be manufactured according to recombinant techniques described herein.

10           In a further embodiment of the invention, the composition of the present invention includes agents useful in wound healing, either by inducing or promoting the formation of tissue, or, alternatively, by limiting the formation of fibrotic adhesions. Such agents include antibiotics, or growth factors, such as connective tissue growth factor, described in, for example, U.S. Patent No. 5,408,040 and 5,585,270, incorporated herein by  
15           reference. In another embodiment of the invention, the drug improves vascularization, for example, tumour necrosis factor, as described in U.S. Patent No. 4,808,402 (issued February 28, 1989).

            With respect particularly to the vascular sealant aspect of the present invention, vascular sealant compositions comprising collagen type III and/or I may be used alone or  
20           in combination with a tissue sealant device, including, for example, the devices set forth in U.S. Patent Nos. 5,782,860 (issued July 21, 1998), 5,759,194 (issued June 2, 1998) and 5,728,132 (issued March 17, 1998).

### Fields Of Use

            The polymerized collagen type III and/or type I products of the present invention  
25           may be useful to produce mechanical sealants and adhesive systems.

**Vascular Adhesive Systems.** Fields of application include, but are not limited to, general surgery, dentistry, neurosurgery, plastic surgery, thorax and vascular surgery, abdominal surgery, orthopaedics, accident surgery, gynaecology, urology, and ophthalmology. The collagen sealants of the present invention have also been used for  
30           local application of drugs, such as antibiotics, growth factors, and cytostatics.

**Sealant Films and Wound Dressings.** In one aspect of the invention, the polymerized collagen products can be made in the form of a sealant film. A collagen-based film will be flexible and elastic with the consistency and feel of plastic film, but can exhibit high biological compatibility. Uses of sealant films include, but are not

5 limited to, prevention of adhesion formation following tendon surgery (i.e., use as a wrap around tendons), use as a synthetic tympanic membrane, and uses as substitute facial tissue and wound dressing components. Additional examples of potential uses of sealant films include, treatment of corneal abrasions, wound closure, coating of catheters and instruments, and use as a material to prevent adhesion formation in tissues and tendons  
10 (e.g., peritoneal cavity).

Further embodiments of the present invention include sealant and adhesive formulations which can be used in systems specific for delivery of numerous drugs and pharmaceutical compositions, including growth factors, antibiotics, and other biologically beneficial compounds. Such materials can be added to the collagen adhesive or sealant to  
15 promote cell migration, cell adhesion, and wound healing.

***Angioplasty and Angiography.*** Angiography is a diagnostic procedure whereby dye is injected into an artery, preferably the femoral artery, to detect the presence or absence of coronary disease. Angioplasty, also known as PCTA, is a therapeutic procedure which involves the inflation of a balloon in an artery, such as the coronary  
20 artery, for the purpose of relieving arterial blockages. After puncturing the femoral artery, a balloon-catheter is introduced through the femoral artery and navigated through to the coronary artery blocked by atherosclerosis (plaque). Once in position, the balloon is inflated and deflated several times in an effort to open the artery by pushing the fatty material against the vessel walls, allowing for blood to circulate to the affected regions of  
25 the heart muscle. Various types of balloon catheters are commonly used in angioplasty and angiography, including over-the-wire catheters which utilize an independent guidewire to the site of the disease; 2) fixed-wire catheters, which combine a balloon catheter with a guidewire into one device; 3) rapid-exchange or single-operator exchange catheters, which are over-the-wire catheters that can be exchanged more conveniently  
30 than standard over-the-wire catheters; and 4) perfusion catheters, which allow blood flow during the procedure. A rotational tip catheter removes plaque buildup on arterial walls. These devices utilize a technique called differential cutting. Calcified material is rendered into microscopic particles without damaging the artery due to the elastic nature of the arterial walls.

5           Angioplasty is a more invasive and complicated procedure than angiography, requiring the insertion of a larger sheath than that used in angiography. The sheath is used as a vehicle for introducing the catheter into the artery. Additionally, angioplasty also requires the use of blood thinners, such as heparin, to prevent clotting during and after the surgical procedure. The anti-clotting agent prevents the body's natural  
10   sealing/clotting mechanism and, thus, sealing punctures requires a significant length of time.

          According to the present invention, after withdrawing the catheter and other invasive devices from the artery, an adhesive applicator may optionally be inserted into the sheath and placed into a position near to or contacting the puncture in the artery.  
15   During the procedure, manual or mechanical pressure is applied to the artery to reduce the flow of blood at the puncture site. If possible, excess blood/fluid is removed from the puncture site. Subsequently, recombinant collagen type III and/or type I monomer of the present invention may be applied to the puncture on the external surface of the artery and/or within the puncture track. The monomer then is polymerized and/or cross-linked  
20   by the techniques described herein, for example, UV irradiation, such that polymerization takes place within 0 to 300 seconds, preferably within 0 to 120 seconds, more preferably within 0 to 30 seconds, and most preferably 3 to 10 seconds. By applying the collagen monomer composition on the outside of the artery, the incidence of embolism (blockage of the artery or circulatory system) is virtually eliminated. Alternatively, polymerization  
25   may be achieved according to the methods set forth in PCT WO 97/29715 and EP 747,066 A2, incorporated herein by reference.

          Alternatively, a polymerized collagen type III and/or type I may be used and the polymerization step may be avoided. Because of the bonding strength of the adhesive of the present invention, only small amounts of the adhesive are required to seal a punctured  
30   artery. Moreover, because the surgical adhesive according to the present invention can polymerize almost immediately, the adhesive can polymerize on the surface and/or along the puncture track of the artery without penetrating the interior of the artery.  
Accordingly, large pieces or particles of material will not enter the circulatory system, thereby substantially reducing risk of embolism. Due to the fast and strong bonding of



- 5 preferred adhesives of the invention, the patient will need to be immobilized for only a minimal period of time.

### Administration

*Formulations.* The tissue treatment composition of the present invention may be presented in the same type of preparations as prior art fibrin sealants. The components  
10 may be provided in deep frozen solution form or as lyophilized powders, to be diluted prior to use with appropriate aqueous solutions, e.g. containing aprotinin and calcium ions, respectively. Additionally, the vascular sealants of the present invention may be formulated and shaped in the form of collagen plugs, as described in the art and known to one of ordinary skill in the art.

15 The compositions of the present invention can additionally comprise pharmaceutical agents, such as, for example, an antibiotic or a growth factor, by incorporating the agent into the tissue adhesive so as to be enclosed in the collagen network formed upon application of the tissue adhesive. The agent is thus kept at the site of application while being controllably released from the composition, such as when the  
20 composition is used as ocular drops, or a wound healing preparation, etc. As also mentioned above, the pharmaceutically active substance to be released from the present tissue adhesive composition may be the viscosity-enhancing polymer in itself or a substance coupled thereto. A specific example of such a viscosity-enhancing polymer fulfilling the viscosity enhancing requirement as well as having therapeutical and  
25 pharmaceutical utility, and in which it may be desired to sustain bioavailability, is hyaluronic acid and salts and derivatives thereof which are easily soluble in water and have an extremely short biological half-life. Thus, in one aspect, compositions of the present invention constitute an advantageous slow-release preparation for proteoglycans such as hyaluronic acid and its salts and derivatives, which considerably increases the  
30 bioavailability thereof.

Notably, the compositions of the present invention are not restricted to those having adhesive properties. Non-adhesive compositions are also included, especially when these compositions are primarily intended for wound healing. These compositions may in particular include non-adhesive proteins such as albumin and/or growth factors.

5 Substantially non-adhesive compositions may also be obtained when the polymer part of the composition inhibits the adhesive properties of the protein part. It should in this context be emphasized that the invention comprises both adhesive and substantially non-adhesive compositions, although it has for simplicity reasons often has been referred to as an "adhesive" in this specification.

10 ***Application Of Compositions.*** The compositions of the present invention may be applied using a variety of dispensing devices. For example, the surgical adhesive may be applied using the devices set forth in U.S. Patent Nos. 4,900,303 (Lemelson) and 5,372,585 (Tiesenbrun) while monitoring the application process through an optical viewing system. The composition of the present invention may also be applied by the  
15 devices set forth in U.S. Patent No. 5,129,882 (Weldon et al.), or the other devices referenced above, or other devices as well known in the art.

Compositions according to the present invention may also be applied in conjunction with other sealing means. For example, adhesive compositions may be applied to puncture sites which have been closed using surgical suture or tape, such as in  
20 the sealing of a puncture or incision in vascular tissues, including the heart. The adhesive in this instance will provide a complete seal, thereby reducing the risk of body fluid leakage from the organ or vessel, e.g., leakage from artery puncture sites. The surgical adhesive of the present invention may additionally be used in conjunction with other sealing means, such as plugs, and the like. Such techniques are set forth in, for example,  
25 U.S. Patent Nos. 4,852,568 (Kensey), 4,890,612 (Kensey), 5,053,046 (Janese), 5,061,274 (Kensey), 5,108,421 (Fowler), 4,832,688 (Sagae et al), 5,192,300 (Fowler), 5,222,974 (Kensey et al.), 5,275,616 (Fowler), 5,282,827 (Kensey et al.), 5,292,332 (Lee), 5,324,306 (Makower et al.), 5,370,660 (Weinstein et al.), and 5,021,059 (Kensey et al.). The subject matter of these patents is incorporated herein by reference.

30 Notably, the compositions of this invention can be used to join together two surfaces by applying the particular composition to at least one of the surfaces. Depending on the particular requirements of the user, the adhesive compositions of this invention can be applied by known means, such as with, for example, a glass stirring rod, sterile brush, or medicine dropper, in many situations, a pressurized aerosol dispensing package is

- 5 preferred in which the adhesive composition is in solution with a compatible anhydrous propellant. Aerosol application of the monomers is particularly advantageous for use in hemostasis. Mechanisms for aerosol applications are well known in the art.

## EXAMPLES

- 10 The following examples are provided solely to illustrate the claimed invention, and are not intended to limit the scope of the invention.

### **Purification of recombinant collagen type III from yeast expression system.**

- 15 The following protocol was used to purify recombinant human collagen type III ("rhC III" or "Rhc III") from *Pichia*.

1. Resuspend 1 volume of cell pellets with 7 volume 0.1N HCL;
2. Fill Bead-Beater chamber half full with glass bead just taken from -20C freezer;
3. Fill the chamber with cell suspension;
- 20 4. Assemble the chamber with Ice-water Jacket;
5. Fill out the jacket with ice water with some sodium chloride;
6. Homogenize the cell pellets for 5 X 1 mins with 5 min. of interval between each 1 min. homogenization;
7. Recover the homogenate by filter through a Buchner Funnel without filter paper;
- 25 8. Add pepsin solution to final concentration of 0.2 mg/ml and incubate for 8 hours at 4°C;
9. Centrifuge for 30 min. at 10,000 rpm and collect the supernant (fraction S1) and pellet (fraction P1);
- 30 10. Adjust the pH to 7.4 with 10M NaOH, Incubate overnight at 4°C;
11. Add 5M NaCl and HAC to 1M NaCl, 0.5M HAC;
12. Incubate at 4°C for 1 hour;
13. Collect the pellets by centrifugation (fraction P2);

- 5           14. Dissolve the pellet in 3 volume 0.1M HCl (depending on the collagen amount, adjust the collagen concentration to about 0.3mg/ml);
15. Add 1.5 volume of 3M urea, 0.3M NaCl, 0.15M Tris, pH 7.4 and adjust the pH to 7.4 with NaOH;
16. Run through DEAE-cellulose column (1.6 X 15 cm) at a flow rate of 0.1-10           0.2ml/min;
17. Collect the flowthrough;
18. Concentrate the collagen by precipitation in 1M NaCl, 0.5M HAC;
19. Redissolve the pellet in 10mM HCl (fraction rhcIII);
20. Dialyze the collagen solution against 10mM HCl if necessary;

15

### Characterization of recombinant human collagen type III

As defined above, purified rhc III was tested by SDS-PAGE as shown in Figure 1 and amino acid analysis was performed and amino acid composition of purified rhc III is shown as below at TABLE 1.

20

TABLE 1

Amino Acid Composition of rhc III Purified from *Pichia Pastoris*

Amino acid	RhC III from <i>Pichia Pastoris</i>	Human Collagen III
Asp	46	42
Glu	68	71
Hyp	132	125
Ser	36	39
Gly	349	350
His	-	6
Arg	42	46
Thr	21	13
Ala	92	96

Pro	105	107
Val	14	14
Met	7	8
Tyr	-	3
Ile	14	13
Leu	20	22
Hyl	0	5
Lys	44	30
Phe	9	8
Hyp/(Hyp+Pro)	0.557	0.538

5

### Biocompatibility and Tissue Response Tests

Biocompatibility and tissue response of rhc III and a commercial was tested in a rat subcutaneous model. Rhc III and commercial available Collagen Hemostat were formulated into injectable paste/gel under sterile condition. The rhc III samples was tested to insure the endotoxin level is below the limit. The gel was injected subcutaneously into rats. The preliminary data indicates that Rhc III does not cause any erythema and edema. The gel was dissected in day 2, 7 and 28 after injection and examined histologically with H&E staining. The commercial available collagen hemostat has a much stronger tissue response than rhc. A comparison of rhc III with the commercial available collagen hemostat harvested from rats on day 7 is shown in Figure 2.

### 20 Platelet Aggregation Test

*Methods.* The fibrillogenesis of above described recombinant human collagen type III ("rchIII") was tested according to the following method: First rhc III solution was diluted with 10mM HCl to 1 mg/ml, next, 1/10 volume of 200mM Na<sub>2</sub>HPO<sub>4</sub>, pH 11.2 was added. The solution was then mixed well and incubated at room temperature (20-22° C) overnight. Following incubation, the fibril slurry was vortexed before usage.

25

5 Human Platelet Rich Plasma (PRP) was then prepared from fresh blood of an apparently healthy donor. The PRP was then adjusted to 200K/ $\mu$ l and the collagen fibril slurry was added into the PRP. Following addition of the fibril slurry, the platelet aggregation profile with an aggregometer was detected. The minimal amount of collagen to induce complete platelet aggregation is estimated from the amount of collagen to  
10 induce a fall in optical density of at least 30% occurred within 5 minutes.

*Experimental Results.* The platelet aggregation capacity of recombinant human collagen III was compared to the platelet aggregation capacity of bovine skin derived collagen according to the methods set forth above and as more fully described in Balleisen, *et al.*, 1975, *Klin. Wschr* 53:903-905, incorporated herein by reference. As set  
15 forth below in TABLE 2, fibrils generated from recombinant human collagen III has a lower minimal amount in inducing human platelet aggregation than fibrils generated from bovine skin collagen. Recombinant human collagen III also has a shorter onset time to induce platelet aggregation. These results indicate that recombinant human collagen is a more hemostatic than tissue derived collagen.

20

TABLE 2

Collagen samples	Minimal Amount for induction of platelet aggregation ( $\mu$ g)	Time to Onset (Second)
Bovine Skin Collagen	10	48
Recombinant human Collagen III from Pichia	<5	36

In a subsequent experiment, platelets were obtained from three healthy donors and were adjusted to 200K/ml in plasma. Collagen fibril slurry was added to the platelet  
25 suspension and the aggregation was measured with an Aggregometer. The minimal amount of collagen capable of inducing complete platelet aggregation was determined by stepwise decreasing the amount of collagen added. It was assumed that complete aggregation had occurred when a fall in OD of at least 30% occurred within 5 minutes. It was shown that rhc III has a lower minimal amount to induce complete aggregation of  
30 platelet, and indicates that rhc III is more hemostatic than bovine collagen I. This results

- 5 depicted in Figure 1 demonstrate that collagen III fibrils are more hemostatic than collagen I fibrils.

### Hemostatic Effects of Rhc III Sponge

Rhc III was prepared as follows:

- 10 Rhc III was expressed in *pichia pastoris*. A selected expression clone was cultivated in a bioreactor under defined conditions. Rhc III was purified from the harvested cell pellets by limited pepsin digestion and differential salt precipitation. Purified rhc III was formulated into fibrils at first by neutralization with phosphate buffer and incubation at room temperature overnight. The fibrils were collected by  
15 centrifugation and then resuspended in water. After homogenization, the rhc III gel was transferred into a mould and lyophilized into a sponge. This type of sponge has very poor water absorption.

- Water absorptive capacity is critical for applications of rhc III as a hemostat and vascular sealant. To ensure the compositions of the present invention could satisfy  
20 this requirement, a process to formulate water absorptive rhc III sponge was developed. Essentially, a sponge was cross-linked, first with UV irradiation and then with 1% WSC. The residual cross-linking reagent was removed by incubation in PBS and the sponge was washed with water. Cross-linked sponges are lyophilized for animal testing and formulation of tissue sealant. This process not only enhanced the  
25 water absorption of rhc III sponge, but also significantly increased the mechanical strength of rhc III sponges. In a control study, bovine collagen I was also formulated into sponges following the same procedures.

- Acclimatized New Zealand White Rabbits were deeply anesthetized and laparotomies were performed to expose the spleens. Using a scapel blade, uniform  
30 incisions about 1.0 cm long and 0.3cm deep were made into the spleens. The incisions were then treated with rhc III sponges or with bovine collagen I sponges. The time interval from the application of the test article or positive control material until the bleeding ceases was recorded. Statistical analysis of the mean time to hemostasis was performed using Anova and Student's two sample T-test. It was observed that the

- 5       bleeding time of spleens treated with rhc III sponges was significantly shorter than that of those treated with bovine collagen I. The results are shown in Figure 4.

### Formulation of RhC III Sealant

To prepare a rhc III sealant, a three-step experimental approach was pursued involving: 1) preparing a cross-linked rhc III sponge; 2) coating the sponge with  
10       human thrombin; and 3) subsequently coating the sponge with human fibrinogen.

A collagen sponge cross-linked with water soluble carbodiimide was rinsed with 100% ethanol and placed in a filtration funnel. Human thrombin suspended in ethanol (25U/ml) was used to coat the sponge by filtration. The amount of thrombin on the sponge was about 10U/cm<sup>2</sup>. Human fibrinogen dissolved in water at  
15       concentration of 4mg/ml was precipitated by mixing with 3 volume of ethanol. The precipitated fibrinogen was filtered through the sponge by vacuum. The amount of fibrinogen on the sponge was about 3mg/ cm<sup>2</sup>. The coated sponge was lyophilized and used for animal tests.

Acclimatized New Zealand White Rabbits were deeply anesthetized and  
20       laparotomies were performed to expose kidneys and spleens. Using a scapel blade, incisions about 1.0 cm long and 0.3cm deep were made into the kidneys and/or spleens. The incision was treated with commercial collagen sponge INSTAT or rhc III sealant. The time interval from the application of the test article until the bleeding ceased was recorded. The adhesive capacity of testing articles was estimated by  
25       peeling the articles from the test site after hemostasis had been achieved. The results are shown in Table 3 and Table 4.

**TABLE 3**

Hemostatic effect and adhesiveness of rhc III Sealant  
in Kidney Injury Model

30

	Non-treatment	Instat <sup>TM</sup>	Rhc III Sealant
Animal Numbers	7	4	4
Bleeding Time (Seconds)	384 +/- 169	67 +/-10	<10 seconds*
Adhesiveness**	ND	+	++

\* Bleeding stopped instantly upon application of the test articles

\*\* + weak, ++ medium, +++ strong.



TABLE 4

Hemostatic Effect and adhesiveness of rhc III Sealant  
in Spleen Injury Model

	Non-treatment	Instat <sup>TM</sup>	Rhc III Sealant
Animal Numbers	7	3	4
Bleeding Time	30.85+/- 9.62 (minutes)	4.27+/-0.47 (minutes)	<10 seconds*
Adhesiveness**	ND	+	++

\* Bleeding stopped instantly upon application of the test articles

\*\* + weak, ++ medium, +++ strong.

The results of this study demonstrated that rhc III fibrils are able to induce human platelet aggregation at a lower concentration than bovine collagen I, indicating that an Rhc III sponge stops bleeding in spleen injury models within a shorter time period than bovine collagen sponges. A prototype of rhc III enhanced fibrin sealant showed a superior hemostatic potential *in vivo* over commercial collagen sponges.

#### Cross-linking Experiments

##### Bovine Collagen Type I

A feasibility cross-linking test was conducted with soluble bovine collagen I. Bovine collagen I in 10mM HCl was neutralized with 1/10 volume of 0.2M Na<sub>2</sub>HPO<sub>4</sub>, pH 11.2 and incubated overnight at room temperature. The fibrils were washed with water and concentrated to 50 mg/ml by centrifugation. The homogenized gel was transferred to a small cell culture dish and lyophilized to form a sponge.

To cross-link the collagen, the bovine collagen sponge was incubated in a solution of water soluble carbodiimide ("WSC") at room temperature overnight, then washed with PBS and water. The cross-linked sponge was then dried and tested for solubility and water absorption, and compared to a commercially available collagen sponge (collagen sponge for Angioseal<sup>TM</sup>). The data is shown in Table 5.

TABLE 5

5

Samples	Color	Solubility in 10mM HCl % of Control	Water Absorption (mg/mg)	Wetting Time (seconds)
Collagen Sponge for Angioseal <sup>TM</sup>	White	1.9%	18.2	5
WSC Treated Vitrogen Sponge				
0%	White	100%	14.5	> 120
0.1%	White	11.7%	18.3	> 120
0.25%	White	2.5%	19.3	100
0.5%	White	0%	19.0	40
1%	White	0%	18.3	22
2%	White	0%	19.8	18

As shown in Table 5 and Figure 5, the cross-linked sponges appeared to be intact after incubation in water for 24 hours at room temperature, while the non-cross-linked collagen sponges were not intact. Using one's fingers, the cross-linked collagen sponges were split to test their mechanical strength. The collagen sponges cross-linked with WSC at a concentration higher than 0.5% demonstrated reasonable mechanical strength.

### Recombinant Collagen Type III

Rhc III collagen fibrils were prepared by neutralizing rhc III in 10mM HCl with 1/10 volume of 0.2M Na<sub>2</sub>HPO<sub>4</sub>, pH 11.2, and incubated overnight at room temperature. Rhc III fibrils were harvested by centrifugation and washed with water. The fibrils were concentrated to 60 mg/ml and homogenized into a gel. The gel was transferred into a mold and lyophilized into a sponge with a thickness of about 2.5mm. The collagen sponge was cross-linked with 1% WSC in 25% ethanol at room temperature for 16 hours. Ethanol was added to accelerate the wetting of collagen sponges in the reaction solution. The cross-linked sponge was then washed with PBS for 2 hours, and subsequently washed three times with water. After lyophilization, collagen sponges of about 1.4 cm<sup>2</sup> in size were used to test for water reabsorption. In addition, 2 mg of the collagen sponge was used to test for solubility. As shown in Table 6, cross-

5 linking of the rhc III sponge improved its water absorptive capacity. The wetting time of the cross-linked rhcIII sponge was reduced to about 10 seconds, and water uptake increased from 76mg to 433mg. The cross-linked rhc III sponge remained intact after incubation in water for 24 hours at room temperature, while the non-cross-linked rhc III did not remain intact.

TABLE 6

Samples	Color	Solubility in 10mM HCl (% of Control)	Water Absorption mg in 90 seconds	Wetting Time (seconds)
Collagen Sponge for TM Angioseal	White	1.9%	450	5
Rhc III Sponge	White	100%	76	> 120
Cross-linked rhc III sponge	White	5.1%	433	10

The collagen sponges were also tested with SDS-PAGE. As shown in Figure 6, lane 1 and lane 6 represent rhc III; lane 2 represents the collagen sponge for  
15 Angioseal™; lane 3 represents cross-linked bovine collagen I sponge; lane 4 represents cross-linked rhc III sponge; and lane 5 represents non-cross-linked rhc III sponge. The collagen sponge Angioseal™, cross-linked bovine collagen I sponge, and cross-linked rhc III sponge is not soluble in SDS-PAGE buffer.

Various modifications and variations of the described methods and systems of the  
20 invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various  
modifications of the described modes for carrying out the invention which are obvious to  
25 those skilled in molecular biology or related fields are intended to be within the scope of the following claims. All references cited herein are incorporated by reference herein in their entirety.

## CLAIMS

5

What is claimed is:

1. A sealant composition comprising a polymerized collagen type III wherein  
10 said sealant composition is produced by recombinantly manufacturing pure collagen type  
III monomers in a cell and polymerizing said monomers with an agent.
2. The composition of claim 1, wherein the composition is biologically  
compatible.
3. The composition of claim 1, wherein the recombinant manufacture of a  
15 collagen type III monomer comprises the following steps:
  - (a) culturing a cell which has been transfected with at least one gene  
encoding a polypeptide comprising collagen type III and at least one gene encoding a  
polypeptide selected from the group the  $\alpha$  or  $\beta$  subunit of prolyl 4-hydroxylase; and
  - (b) purifying said collagen type III.
- 20 4. The composition of claim 1, wherein the composition is further comprised  
of one or more agents selected from the group fibrin, fibrinogen, thrombin, Factor XIII,  
or connective tissue growth factor.
5. The composition of claim 1, wherein the sealant is a vascular sealant.
6. The composition of claim 1, wherein the collagen is gelatin.
- 25 7. The composition of claim 1, wherein the composition is non-adhesive.
8. The composition of claim 7, wherein the composition is further comprised  
of albumin.
9. The composition of claim 1, wherein the polymerizing is accomplished by  
cross-linking.
- 30 10. A process for making a tissue sealant comprising the steps:
  - (a) manufacturing collagen type III monomers by recombinant means;  
and
  - (b) polymerizing said collagen type III monomers.

- 5           11.     The process of claim 10, wherein the polymerizing is accomplished by cross-linking.
12.     A sealant composition comprising a polymerized collagen type I wherein said sealant composition is produced by recombinantly manufacturing pure collagen type I monomers in a cell and polymerizing said monomers with an agent.
- 10          13.     The composition of claim 12, wherein the composition is biologically compatible.
14.     The composition of claim 12, wherein the recombinant manufacture of a collagen type I monomer comprises the following steps:
- (a)     culturing a cell which has been transfected with at least one gene
- 15     encoding a polypeptide comprising collagen type I and at least one gene encoding a polypeptide selected from the group the  $\alpha$  or  $\beta$  subunit of prolyl 4-hydroxylase; and
- (b)     purifying said collagen type I.
15.     The composition of claim 12, wherein the collagen is a gelatin.
16.     The composition of claim 12, wherein the composition is further
- 20     comprised of one or more agents selected from the group fibrin, fibrinogen, thrombin, Factor XIII or connective tissue growth factor.
17.     The composition of claim 12, wherein the sealant is a vascular sealant.
18.     The composition of claim 12, wherein the collagen type I is a heterotrimer collagen.
- 25          19.     The composition of claim 12, wherein the collagen type I is a homotrimer collagen.
20.     The composition of claim 12, wherein the composition is non-adhesive.
21.     The composition of claim 20, wherein the composition is further comprised of albumin.
- 30          22.     The composition of claim 12, wherein the polymerizing is accomplished by cross-linking.
23.     A process for making a tissue sealant comprising the steps:
- (a)     manufacturing collagen type I monomers by recombinant means;
- and

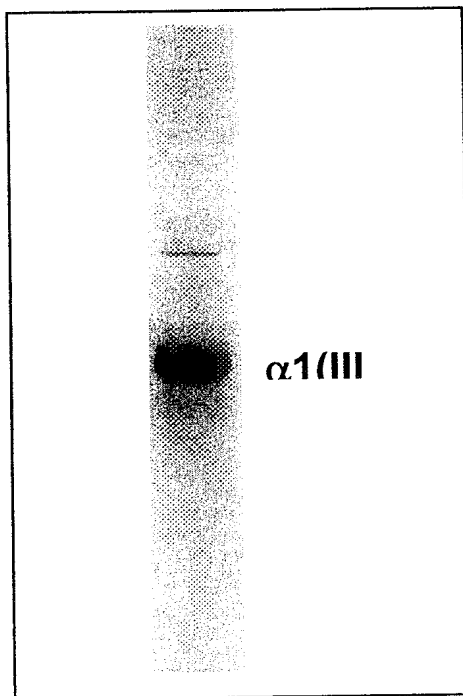
- 5 (b) polymerizing said collagen type I monomers.
24. The process of claim 23, wherein the polymerizing is accomplished by cross-linking.
25. A tissue sealant composition comprising a polymerized pure collagen type III and a polymerized pure collagen type I.
- 10 26. The composition of claim 25 wherein the composition is biologically compatible.
27. The composition of claim 25 wherein the ratio of pure recombinant collagen type III to pure recombinant collagen type I is about 30% or greater collagen type III to about 70% or less collagen type I.
- 15 28. The composition of claim 25 wherein the collagen is a gelatin.
29. The composition of claim 25 wherein the composition is further comprised of one or more agents selected from the group fibrin, fibrinogen, thrombin, Factor XIII or connective tissue growth factor.
30. A wound dressing composition comprising a polymerized collagen type III wherein said composition is produced by recombinantly manufacturing pure collagen type III monomers in a cell and polymerizing said monomers with an agent.
- 20 31. The composition of claim 30 wherein the composition is biologically compatible.
32. The composition of claim 30 wherein the recombinant manufacture of a collagen type III monomer comprises the following steps:
- 25 (a) culturing a cell which has been transfected with at least one gene encoding a polypeptide comprising collagen type III and at least one gene encoding a polypeptide selected from the group the  $\alpha$  or  $\beta$  subunit of prolyl 4-hydroxylase; and
- (b) purifying said collagen type III.
- 30 33. The composition of claim 30, wherein said collagens are a gelatin.
34. The composition of claim 30, wherein the composition is further comprised of one or more agents selected from the group fibrin, fibrinogen, thrombin, Factor XIII or connective tissue growth factor.

- 5           35.    The composition of claim 30, wherein the polymerizing is accomplished by cross-linking.
36.    A process for making a wound dressing comprising the steps:
- (a)    manufacturing collagen type III monomers by recombinant means;
- and
- 10           (b)    polymerizing said collagen type III monomers.
37.    The process of claim 36, wherein the polymerizing is accomplished by cross-linking.
38.    A wound dressing composition comprising a polymerized collagen type I wherein said composition is produced by recombinantly manufacturing pure collagen
- 15           type I monomers in a cell and polymerizing said monomers with an agent.
39.    The composition of claim 38 wherein the composition is biologically compatible
40.    The composition of claim 38 wherein the recombinant manufacture of a collagen type I monomer comprises the following steps:
- 20           (a)    culturing a cell which has been transfected with at least one gene encoding a polypeptide comprising collagen type I and at least one gene encoding a polypeptide selected from the group the  $\alpha$  or  $\beta$  subunit of prolyl 4-hydroxylase; and
- (b)    purifying said collagen type I.
41.    The composition of claim 38 wherein the said collagen is a gelatin.
- 25           42.    The composition of claim 38 wherein the composition is further comprised of one or more agents selected from the group fibrin, fibrinogen, thrombin, Factor XIII or connective tissue growth factor.
43.    The composition of claim 38, wherein the collagen is gelatin.
44.    The composition of claim 38, wherein the collagen type I is a heterotrimer
- 30           collagen.
45.    The composition of claim 38, wherein the collagen type I is a homotrimer collagen.
46.    The composition of claim 38, wherein the polymerizing is accomplished by cross-linking.

- 5           47.    A process for making a wound dressing comprising the steps:  
              (a)    manufacturing collagen type I monomers by recombinant means;  
              and  
              (b)    polymerizing said collagen type I monomers.
48.    The process of claim 47, wherein the polymerizing is accomplished by  
10   cross-linking.
49.    A wound dressing composition comprising a polymerized pure collagen  
          type III and a polymerized pure collagen type I.
50.    The composition of claim 49 wherein the composition is biologically  
          compatible.
- 15           51.    The composition of claim 49, wherein the ratio of pure recombinant  
          collagen type III to pure recombinant collagen type I is about 30% or greater collagen  
          type III to about 70% or less collagen type I.
52.    The composition of claim 49, wherein said collagens are gelatin.
53.    The composition of claim 49, wherein the composition is further  
20   comprised of one or more agents selected from the group fibrin, fibrinogen, thrombin,  
          Factor XIII or connective tissue growth factor.

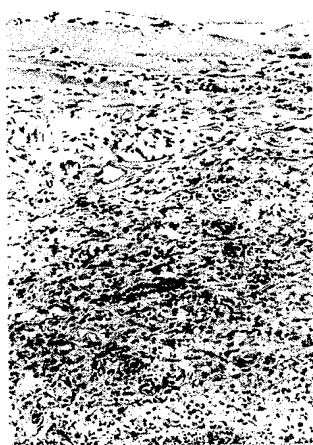


Figure 1



SDS-PAGE of RhC III from *Pichia*

Figure 2



Commercial Collagen Hemostat



RhC III from *Pichia Pastoris*

Figure 3

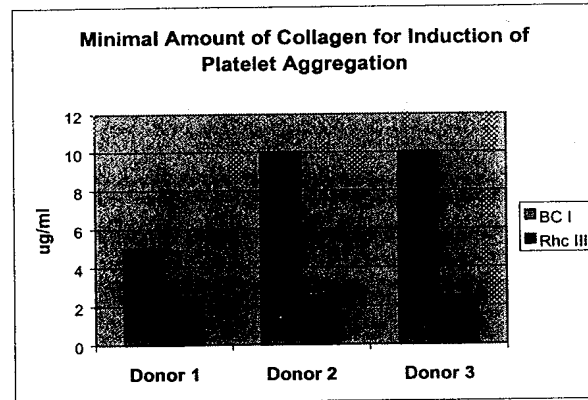


Figure 4

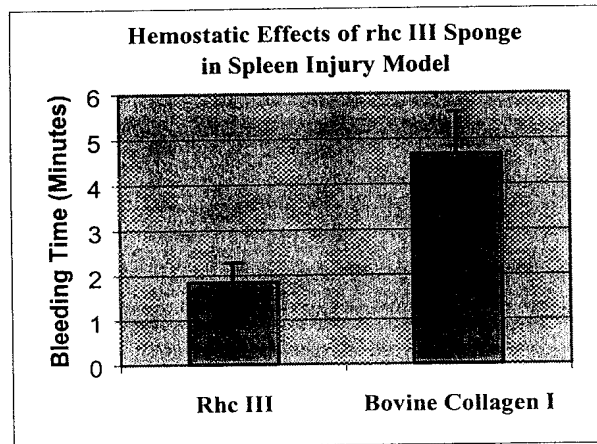


Figure 5

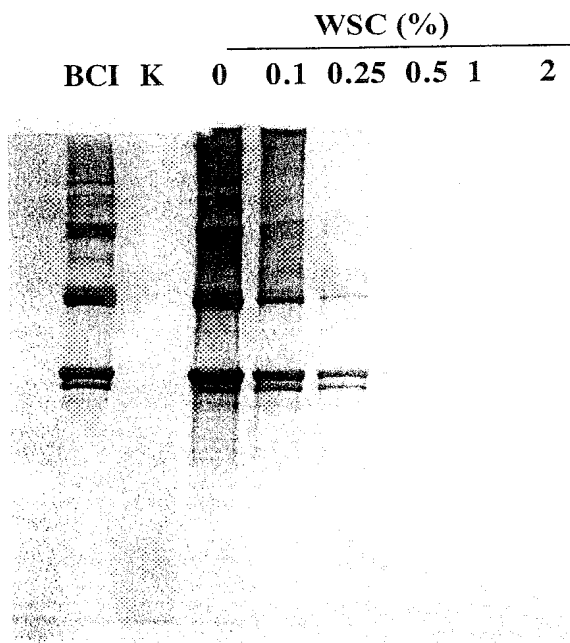
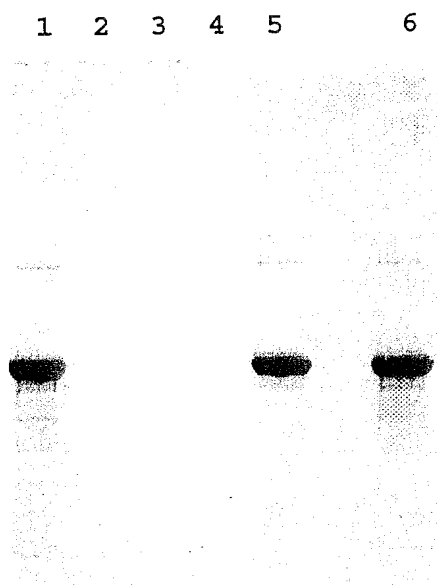


Figure 6



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/18095

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61B 17/04, 17/08, 17/12

US CL : 128/325; 606/214

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 128/325; 606/214

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
NONE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,852,568 A (KENSEY) 01 August 1989, see entire document.	1-53
Y	US 5,759,194 A (HAMMERSLAG) 02 June 1998, see entire document.	1-53



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*P* document published prior to the international filing date but later than the priority date claimed	

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16 DECEMBER 1999

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11 JAN 2000

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